

## **Humoral Immune Response to Mesothelin in Mesothelioma and Ovarian Cancer Patients**

Mitchell Ho, Raffit Hassan, Jingli Zhang, Qing-cheng Wang, Masanori Onda, Tapan Bera, and Ira Pastan

**Abstract** **Purpose:** Mesothelin is a glycosyl-phosphatidylinositol – anchored glycoprotein present on the cell surface. Mesothelin is a differentiation antigen that is highly expressed on mesothelioma, ovarian cancer, and pancreatic cancer. The existence of a spontaneous humoral immune response to mesothelin in humans has not been fully studied. Here we addressed the issue of whether mesothelin elicits a humoral immune response in patients with mesothelioma and ovarian cancer.

**Experimental Design:** Using an ELISA, we analyzed immunoglobulin G antibodies specific for mesothelin in sera from patients with mesothelioma and epithelial ovarian cancer. Tumor specimens were examined by immunohistochemistry for mesothelin protein expression.

**Results:** Elevated levels of mesothelin-specific antibodies were detected in the sera of 39.1% of patients with mesothelioma (27 of 69 patients) and 41.7% with epithelial ovarian cancer (10 of 24 patients) when compared with a normal control population (44 blood donors;  $P < 0.01$  for both mesothelioma and ovarian cancer). We also found that 53% to 56% of patients with mesothelin immunostaining-positive mesothelioma and ovarian cancer had antibodies specific for mesothelin, whereas only 0% to 8% of patients with negative mesothelin immunostaining had detectable mesothelin-specific antibodies ( $\chi^2$  test:  $P < 0.01$  for mesothelioma and  $P = 0.025$  for ovarian cancer).

**Conclusions:** Our findings indicate that mesothelin is a new tumor antigen in patients with mesothelioma and ovarian cancer and the immunogenicity of mesothelin is associated with its high expression on the tumor cells. Mesothelin represents an excellent target for immune-based therapies.

The search for human tumor antigens eliciting immune responses in an autologous host has been a long-standing challenge in tumor immunology. The growing list of tumor antigens recognized by B cells or cytotoxic T cells provides attractive targets for vaccine-based approaches to cancer immunotherapy (reviewed in ref. 1).

Mesothelin is a glycosyl-phosphatidylinositol – anchored glycoprotein present on the cell surface. Mesothelin was first defined by the monoclonal antibody (mAb) K1 (2). The hybridoma producing K1 was isolated from the spleens of mice immunized with an ovarian cancer cell line (OVCAR-3). The mesothelin gene encodes a 71-kDa precursor protein that is processed to a 40-kDa membrane-bound protein termed mesothelin and a 31-kDa shed fragment called megakaryocyte-potentiating factor that is released from the cell (2, 3). The biological functions of mesothelin remain speculative. It was suggested that mesothelin might have a role in adhesion (2, 4). Mutant mice with targeted mesothelin gene inactivation are

normal, exhibiting no apparent anatomic, hematologic, or reproductive abnormalities (5). Mesothelin is a differentiation antigen present at low levels on a restricted set of normal adult tissues but aberrantly overexpressed in mesotheliomas, ovarian cancers, and some other cancers (3). The limited distribution of mesothelin on normal tissues makes it a promising target for tumor-specific therapy. Antimesothelin recombinant SS1(dsFv)PE38, or SS1P, the antibody-toxin chimeric protein composed of a Fv portion of SS1 (6) and a truncated form of *Pseudomonas* exotoxin, has significant antitumor activity against mesothelin-positive tumor cells and is being evaluated in phase I studies (3).

There is experimental evidence to suggest that mesothelin is a strongly immunogenic protein. In patients with pancreatic cancer who were vaccinated with granulocyte macrophage colony-stimulating factor-secreting pancreatic tumor cell lines, a dose-dependent systemic antitumor immunity against autologous tumors was seen. All patients who had this benefit had a strong mesothelin-specific CD8<sup>+</sup> T-cell immune response (7). The existence of a spontaneous B-cell immune response to this tumor antigen in cancer patients requires investigation. By Western blot, Suarez-Alvarez et al. found that circulating immunoglobulin G antibodies to mesothelin were detected in only 2 of 50 (4%) patients with pharynx/larynx squamous cell carcinoma (8). This tumor type has much less intense mesothelin expression compared with mesothelioma and ovarian cancer (9).

In the present work, we addressed the issue of whether mesothelin, a differentiation antigen highly expressed in

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mesothelioma and ovarian cancer, elicits a humoral immune response in these cancer patients. By ELISA, we found that mesothelin-specific immunoglobulin G antibodies were elevated in the sera from these patients compared with a normal control population. Furthermore, analysis of mesothelin immunostaining in mesothelioma and ovarian tumor cells indicates that a high percentage of anti-mesothelin antibodies in patient sera is associated with high expression of this tumor antigen.

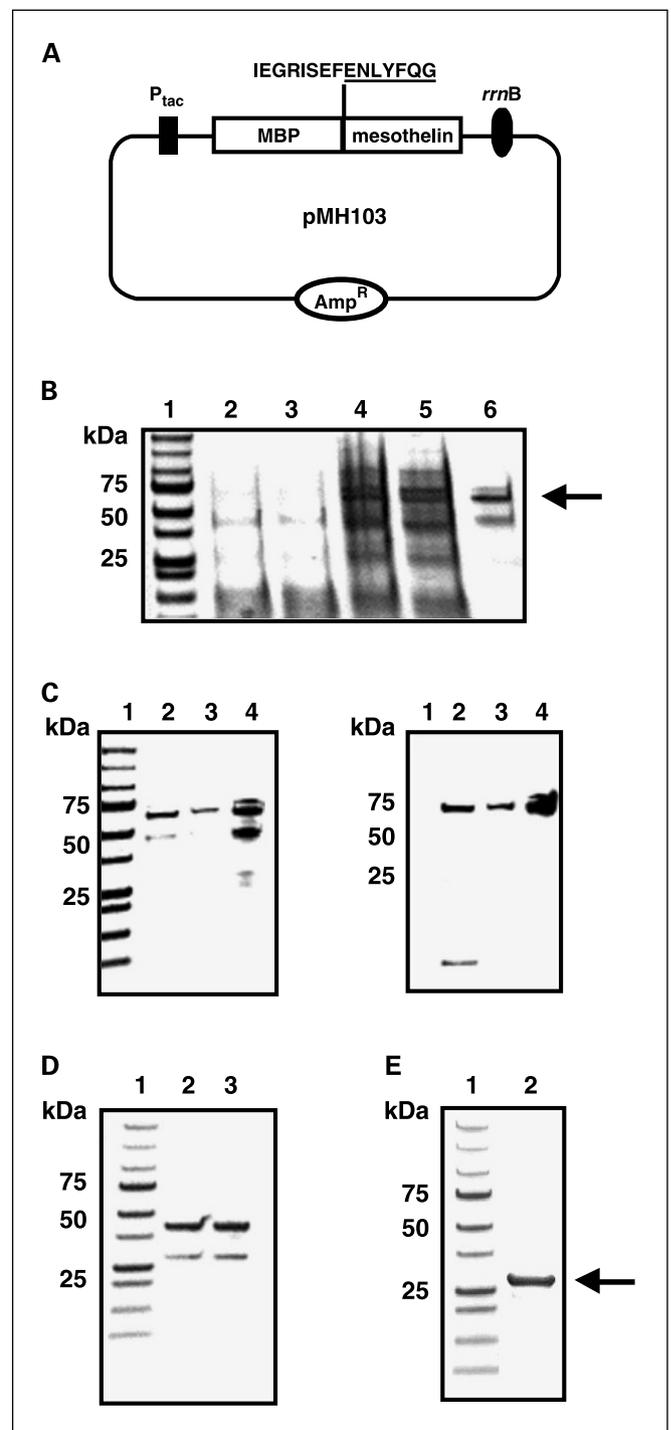
## Materials and Methods

**Human subjects.** Peripheral blood was obtained from patients who were evaluated for possible enrollment in the clinical trials of the anti-mesothelin immunotoxin, SS1P. The SS1P studies were approved by the National Cancer Institute Institutional Review Board and patients signed an informed consent. Sera from 24 patients with ovarian cancer and 69 patients with mesothelioma were evaluated in this study. The median age of patients with ovarian cancer was 63 years (range, 37-80 years). The median age of 69 patients (49 males and 20 females) with mesothelioma was 61 years (range, 27-80 years). Forty-four serum samples from apparently healthy blood donors obtained from Bioreclamation (East Meadow, NY) were used as controls. Blood donors averaged  $36.05 \pm 11.01$  years of age, and males and females were equally represented.

**Mesothelin expression and purification.** To construct the maltose-binding protein (MBP)-mesothelin fusion expression plasmid, pMH103, the gene encoding mesothelin was first PCR-amplified from IMAGE human cDNA clone ID 5209096 using primers MesoMatMalF (5'-TCTTCTCTCGAATTCGAAAACCTGTATTTTCAGGGCGAAGTGGAGAAGACAGCC-3') and MesoMalR (5'-TCTTCTCTCAAGCTTGCTTAGCCCTGTAGCCCCAGCCC-3'). The primer sequence in bold encodes a tobacco etch virus protease cleavage site (ENLYFQG). The cloned mesothelin gene is flanked by 5' *Eco*RI and 3' *Hin*DIII sites to facilitate in-frame cloning of mesothelin genes into pMAL-p2X (NEB, Beverly, MA). The resulting plasmid pMH103 encodes a fusion protein consisting of the *malE* signal sequence, *malE*, and mesothelin. The *malE* gene of *Escherichia coli* encodes MBP (Fig. 1A). The fusion protein is directed to the periplasm of *E. coli*.

MBP-mesothelin protein was produced in BL21-CodonPlus-RIL (Stratagene, La Jolla, CA) *E. coli* containing pMH103 and purified according to the manufacturer's instructions (NEB). As shown in Fig. 1B, a high level of expression of an induced protein of 74 kDa was achieved 2 hours after isopropyl  $\beta$ -D-thiogalactopyranoside induction. This is in agreement with the expected molecular mass of the fusion protein MBP (42 kDa) and mesothelin without glycosylation (32 kDa). The purified MBP-mesothelin fusion proteins were analyzed by SDS-PAGE (Fig. 1C, left). This 74-kDa protein strongly reacts with mAb K1 (Fig. 1C, right). After recombinant tobacco etch virus cleavage (Invitrogen, Carlsbad, CA), mesothelin and MBP were separated as shown on SDS-PAGE: mesothelin (~32 kDa) and MBP (~42 kDa; Fig. 1D). The collected samples were then applied to an amylose column for removal of MBP (Fig. 1E). Alternatively, we also used a monoQ column to separate the mesothelin proteins from the MBP. The purity of mesothelin at the final step was over 95%.

**ELISA.** Ninety-six-well microtiter plates (Maxi-sorb, NUNC, Rochester, NY) were coated with 0.5  $\mu$ g/mL purified recombinant mesothelin or MBP, or 2  $\mu$ g/mL purified rabbit Fc-mesothelin fusion proteins, in 50 mmol/L bicarbonate buffer (pH 9.5) at 4°C overnight, blocked with 1% of bovine serum albumin in PBS at room temperature for 1 hour, and washed with PBS containing 0.05% Tween 20 (PBST). Normal or patient sera (50  $\mu$ L) diluted 1:100 in PBST containing 1% bovine serum albumin, were added to precoated wells. After 1 hour at room temperature, the plates were washed four times. Each well was then incubated for 1 hour with 50  $\mu$ L of a 1:6,000 dilution of goat antihuman immunoglobulin G F(ab)<sub>2</sub> labeled with horseradish



**Fig. 1.** A, diagram of expression plasmid pMH103 for mesothelin expression in *E. coli* as NH<sub>2</sub>-terminal fusions to MBP. The sequence above the junction of MBP and mesothelin indicates the amino acid sequence of the linker joining the two regions. The underlined sequence is the tobacco etch virus cleavage site. P<sub>tac</sub>, tac inducible promoter; *rrnB*, transcription terminator; *Amp*<sup>R</sup>, ampicillin resistance gene. B, analysis of mesothelin expression. Location of the fusion protein band (arrowhead). Lane 1, molecular weight markers; lanes 2 and 3, transformed cells before IPTG induction; lanes 4 and 5, transformed cells after IPTG induction; lane 6, fluid from osmotic shock of transformed cells after IPTG induction. C, purification of the fusion protein MBP-mesothelin using an amylose affinity column. SDS-PAGE (left); Western blotting with anti-mesothelin mAb K1 (right). Lane 1, molecular weight standards; lane 2, fraction eluted from the amylose column with SDS; lane 3, main fraction eluted with 10 mmol/L maltose; lane 4, osmotic shock fluid. D, recombinant tobacco etch virus cleavage of MBP-mesothelin proteins. Lane 1, molecular weight; lanes 2 and 3, mesothelin and MBP. E, removal of the MBP moiety. Lane 1, molecular weight; lane 2, purified mesothelin (arrowhead) in flow-through from an amylose column.

peroxidase (Jackson ImmunoResearch, West Grove, PA) at room temperature. Assays were developed with tetramethyl benzidine/H<sub>2</sub>O<sub>2</sub> substrate (Pierce, Rockford, IL). The absorbance was read at 450 nm. The assay was initially done with serial dilution of individual serum samples from 1:10 to 1:10,000 to confirm that measurements were representative of a specific antigen-antibody affinity reaction. All serum samples were run in duplicate and randomly distributed on the plates. Sera from cancer patients and sera from control blood donors were tested simultaneously.

Binding of rabbit anti-mesothelin antibodies and chimeric protein SS1P immunotoxin specific for mesothelin was done on mesothelin essentially as described above. Binding of anti-mesothelin antibodies from immunized rabbits was detected by 1:5000 dilution of horseradish peroxidase-labeled goat F(ab)<sub>2</sub> anti-rabbit immunoglobulin G (Biosource, Camarillo, CA). Binding of SS1P to mesothelin was detected by a rabbit anti-*Pseudomonas* exotoxin antibody (5 µg/mL) followed by 1:5,000 dilution of horseradish peroxidase-labeled goat F(ab')<sub>2</sub> anti-rabbit immunoglobulin G (Biosource).

**Immunohistochemistry.** Mesothelin expression in tumor specimens was evaluated by a pathologist following the immunohistochemistry protocols using the anti-mesothelin mAbs K1 (10) or 5B2 (11). Mesothelin positivity was identified by brownish staining of the surface of the tumor cells and graded as positive if at least 30% of accessible tumor cells were labeled.

**Transfection and flow cytometric analysis.** Flow cytometric analysis was done to measure the reactivity of human sera to membrane-bound mesothelin at the cell surface. A431 human carcinoma cells were transfected with pcDNA3.1(+) (Invitrogen; mock), or pMH107, a pcDNA3.1(+) vector containing a full-length mesothelin cDNA by LipofectAMINE (Invitrogen). We obtained a homogeneous A431 cell line (A431.H9) stably expressing mesothelin by single cell sorting with a FACSVantage SE (BD Biosciences, San Jose, CA). In a typical protocol for flow cytometric analysis,  $5 \times 10^5$  cells were incubated with 1:100 dilution of human sera in 250 µL of PBS containing 5% bovine serum albumin and 0.1% sodium azide. After incubation for 1 hour at 4°C, the cells were washed once with the same buffer and incubated with 1:100 dilution of FITC-labeled goat F(ab)<sub>2</sub> anti-human immunoglobulin G (Biosource) for 1 hour. After washing twice, the cells were suspended in 0.5 mL of PBS, and the fluorescence associated with the live cells was measured using a FACSCalibur flow cytometer (BD Biosciences).

Binding of SS1P to A431.H9 cells were done essentially as described above but with purified recombinant immunotoxin proteins SS1P or HA22 (anti-CD22 immunotoxin proteins; ref. 12; control; 10 µg/mL) and a rabbit anti-*Pseudomonas* exotoxin antibody (10 µg/mL) detected by 1:100 dilution of FITC-labeled goat F(ab)<sub>2</sub> anti-rabbit immunoglobulin G (Biosource).

**Western blot analysis.** The recombinant MBP-mesothelin fusion proteins (2 µg per lane) were separated by a 4% to 20% Tris-glycine SDS-PAGE and electroblotted onto nitrocellulose filters. The filters were incubated sequentially with primary mAb K1 at 1:500 and horseradish peroxidase-conjugated goat anti-mouse immunoglobulin G (Biosource) at 1:20,000. Proteins were visualized by the enhanced chemiluminescence kit (Amersham Biosciences, Piscataway, NJ).

**Preparation of rabbit Fc-mesothelin fusion proteins.** The mesothelin protein was expressed as a fusion to rabbit immunoglobulin G Fc in transfected 293T cells. The DNA fragments encoding rabbit immunoglobulin G Fc were amplified by PCR using the plasmid pyB1-12,14 for the rabbit Fc (amino acids 96-323, Swissprot: P01870, kindly provided by Dr. Rose G. Mage, NIH) as the template and inserted into pSecTag2 (Invitrogen). The resulting plasmid pOND-rFc-Meso contains the same cDNA for mesothelin (amino acids 296-580) as in pMH103. The rabbit Fc mesothelin fusion proteins were harvested from the culture supernatant and purified with Hi-Trap protein A column (Amersham Biosciences).

**Statistical analysis.** The data obtained was entered in Prism (version 3.02) for Windows (GraphPad Software, San Diego, CA) for statistical

analysis. ELISA raw data were analyzed by ANOVA with Dunnett's and Newman-Keuls multiple comparison post tests.  $P_s < 0.05$  were considered statistically significant.

## Results

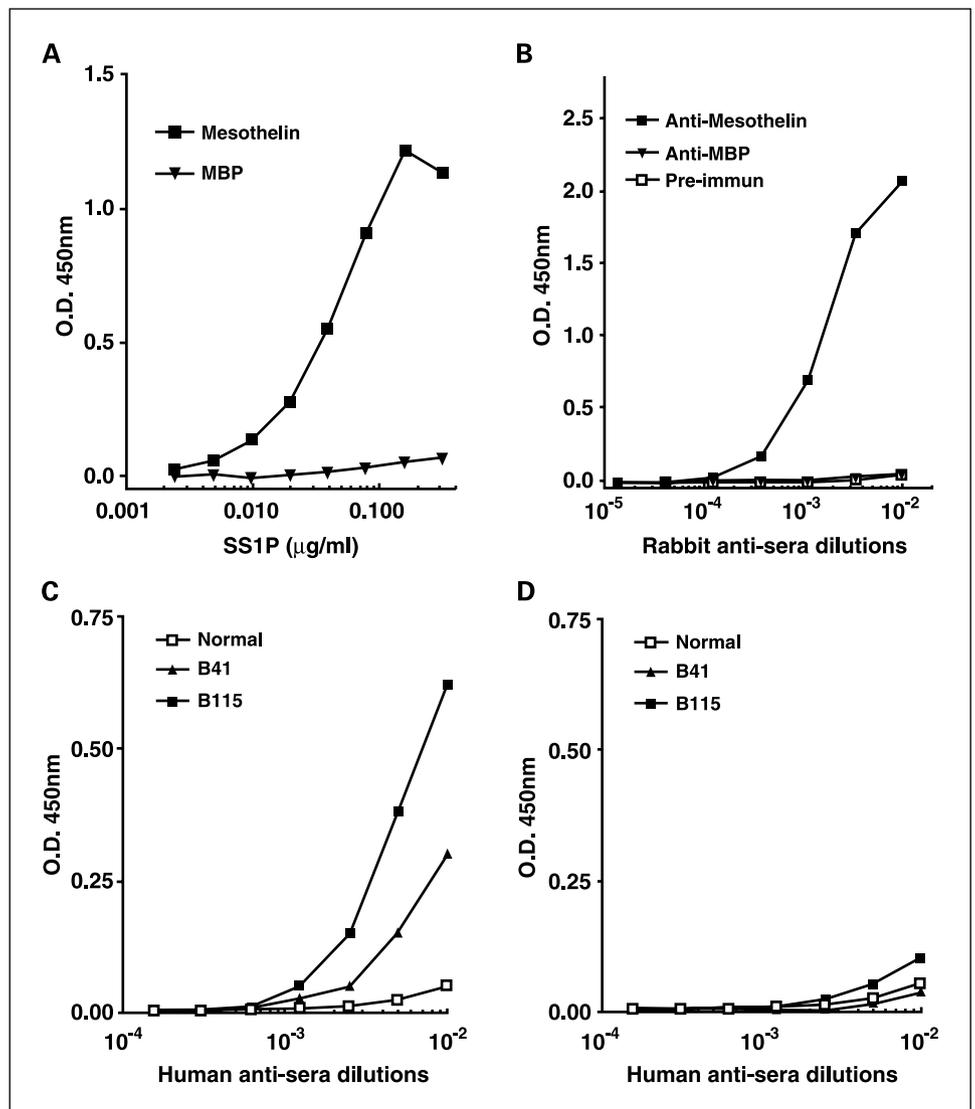
### *Expression and purification of recombinant human mesothelin.*

The gene encoding mesothelin was obtained from IMAGE human cDNA EST clone ID 5209096. The full-length cDNA sequence has been deposited in Genbank with accession number AY743922. The mesothelin cDNA is 2,187-bp long, contains an open reading frame of 1,869 bp, and encodes a protein of 622 amino acids. The mesothelin gene encodes a precursor protein that is processed to the MPF fragment (amino acids 34-286) and the mesothelin protein (amino acids 296-622) attached to the cell membrane by a glycosyl-phosphatidylinositol linkage (3). By analyzing EST databases, we have observed that this is the most common mesothelin variant. Thus, we decided to use this sequence in the present study. To produce the soluble mesothelin protein without the MPF portion, we expressed a recombinant form of mesothelin (amino acids 296-580). We chose to express mesothelin truncated at Gly-580. We predicted that the potential cleavage site for glycosyl-phosphatidylinositol attachment is located at Ser<sup>598</sup> using the DGPI program (<http://129.194.185.165/dgpi/>). Thus, the truncation should completely remove the region of the COOH-terminal hydrophobic domain that is essential for attachment of the glycosyl-phosphatidylinositol anchor. The purity of mesothelin is over 95% (Fig. 1E).

**Establishment of an ELISA system for detecting anti-mesothelin antibodies.** To determine if we could detect antibodies to mesothelin by ELISA, we coated plates with mesothelin or a control protein, MBP, and added immunotoxin SS1P that contains a high-affinity Fv specific for mesothelin (Fig. 2A). Both mesothelin and MBP used in this study were purified from the same *E. coli* expression and purification system so should contain similar contaminants. We found that SS1P bound mesothelin but not MBP in ELISA. We also used Western blot analysis (Fig. 1C) to show that SS1P and a mAb to mesothelin (K1) recognized the recombinant mesothelin protein and not the control MBP. In a third series of specificity experiments, we employed anti-sera from rabbits immunized with a mesothelin expression plasmid (13). We incubated sera collected from rabbits before and after DNA immunization with a pcDNA3.1(+) vector containing the mesothelin cDNA (pMH107; Fig. 2B). The immunized rabbit anti-sera showed specificity for mesothelin and did not cross-react with MBP. The normal rabbit sera did not bind mesothelin.

These three sets of data show that the ELISA system can detect antibodies specific for mesothelin in sera. Next we used two representative patient sera, B115 and B41, for standardizing and optimizing conditions for ELISA (e.g., conditions for antigen coating, blocking, and washing). As shown in Fig. 2C, patient sera B115 and B41 bound mesothelin but did not bind MBP (Fig. 2D). B115 and B41 were sera from patients with peritoneal mesothelioma. The ELISA signals of B115, B41 and pooled normal control sera were 0.62, 0.3, and 0.05 at 1:100 dilutions; 0.38, 0.15, and 0.02 at 1:200 dilutions; and 0.15, 0.05, and 0.014 at 1:400 dilutions. Sensitivity was best when sera was used at 1:100 dilutions. The signal-to-background ratios were better when sera was between 1:100 and 1:200

**Fig. 2.** *A*, reactivity of immunotoxin protein SS1P against mesothelin. *B*, reactivity of rabbit anti-sera against mesothelin. Sera collected from rabbits before (*Preimmune*) and after DNA immunization with the mesothelin cDNA were incubated with mesothelin or MBP. *C*, immunologic activities of mesothelin. Two representative patient sera B115 and B41 and pooled normal blood donors (*Normal*) were incubated with mesothelin or MBP (*D*) recombinant proteins. Absorbance was read at 450 nm.

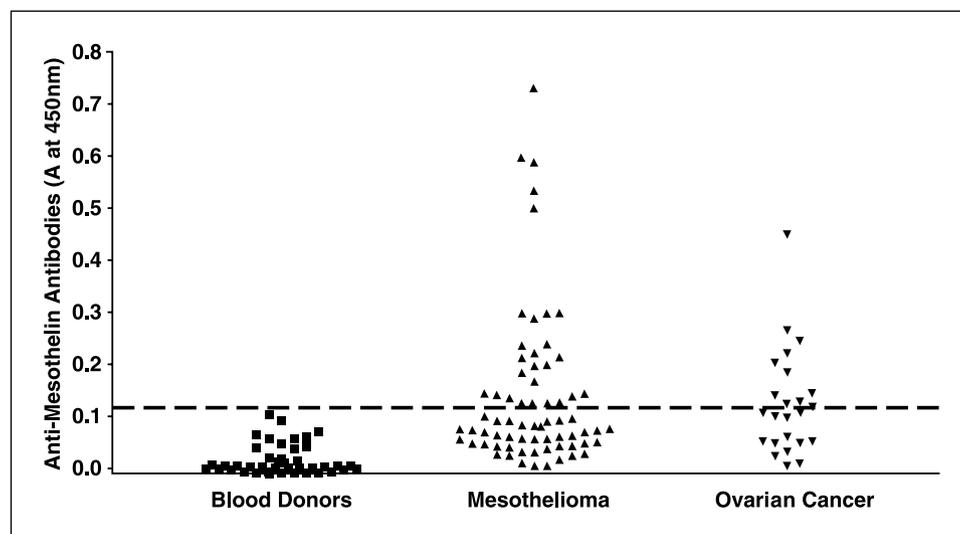


dilutions. Therefore, we decided to use a 1:100 dilution of sera in ELISA to achieve the high sensitivity and good signal-to-background ratios.

**Specific immunoglobulin G response to mesothelin in patients with cancer.** Using the ELISA described above, we detected and quantified the levels of specific immunoglobulin G to mesothelin present in the sera of patients with mesothelioma and ovarian cancer. A positive reaction is defined as an absorbance value of a 1:100 diluted serum that exceeds the mean absorbance value of sera from normal donors ( $n = 44$ ) by three SDs (0.103). We chose to use this ELISA definition (an absorbance value  $>3$  SDs above the mean absorbance value of sera of normal donors) for positive antibody reactivity because it has been commonly used by other investigators to describe the humoral immune response to other tumor antigens such as NY-ESO-1, MAGE-1, MAGE-3, SSX2, survivin, and HOXA7 (14–16). As summarized in Fig. 3, antibodies were not detected in normal donors ( $n = 44$ ), but specific mesothelin reactivity was detected in sera from 39.1% of patients with mesothelioma (27 of 69 patients) and 41.7% of ovarian cancer (10 of 24 patients). In each instance, specificity was confirmed by showing that ELISA reactivity was blocked by prior

incubation of sera with excess purified mesothelin proteins (data not shown). We also found that in two to four serum samples collected from each patient at different dates had similar reactivity. The average (mean  $\pm$  SE) of mesothelin-specific antibody absorbance read spectrophotometrically at 450 nm was  $0.137 \pm 0.018$  for mesothelioma patient sera and  $0.122 \pm 0.021$  for ovarian cancer patient sera. Anti-mesothelin antibody response in both cancer patients was significantly elevated above the mean of normal control sera ( $0.015 \pm 0.004$ ;  $P < 0.01$ ). A statistically significant difference in anti-mesothelin antibody responses between mesothelioma and ovarian cancer patients was not observed in this study ( $P > 0.05$ ). Neither normal nor patient antibody measurements correlated with age, sex, or ethnic background ( $P > 0.1$  for all comparisons). These results confirm the specificity of the response to mesothelin in these patients and suggest that mesothelin was capable of eliciting a humoral immune response in patients with mesothelioma and ovarian cancer.

As shown in Table 1, we also found that among 49 patients with mesothelioma highly expressing mesothelin as measured by immunostaining with anti-mesothelin mAb K1 or 5B2, 26 patients (53%) elicited high humoral immune response



**Fig. 3.** Anti-mesothelin antibodies detected by ELISA in sera at a dilution of 1:100 from patients with mesothelioma ( $n = 69$ ) and ovarian cancer ( $n = 24$ ) in comparison with sera from normal blood donors (control population,  $n = 44$ ). Values are expressed as absorbance. The horizontal dashed line indicates the upper limit of the negative control (3 SD above the absorbance mean of total normal donor sera;  $>0.103$ ).

against mesothelin. In contrast, among 13 patients with immunostaining negative mesothelioma, only one patient (8%) had a low but detectable antibody response (absorbance, 0.213;  $\chi^2$  test,  $P < 0.01$ ). This observation may indicate the lower sensitivity of immunohistochemistry for detection of mesothelin compared with ELISA for detection of the anti-mesothelin antibodies. It is possible that the tumor samples were poorly preserved; therefore, the antibody used for immunohistochemistry did not recognize the antigen. Similarly, among 18 patients with immunostaining-positive ovarian carcinomas, 10 of them (56%) elicited high humoral response specific for mesothelin whereas none of six patients (0%) with immunostaining negative ovarian carcinomas had anti-mesothelin antibody response ( $\chi^2$  test,  $P = 0.025$ ).

**Confirmation of the specificity of the antibody response.** The specificity of the reactivity of the patient sera against mesothelin was confirmed by flow cytometry and ELISA using mesothelin from different sources. For the fluorescence-activated cell sorting experiments, a new A431 cell line (A431.H9) stably expressing mesothelin was established by transfection of cells with pMH107 containing the mesothelin cDNA. To verify mesothelin expression on this cell line, flow cytometry analysis was carried out with chimeric protein immunotoxin SS1P. The data in Fig. 4A show that SS1P, but not the CD22-specific immunotoxin HA22, binds to mesothelin on the surface of the A431.H9 cells. There was no signal of SS1P with control A431 cells transfected with an empty pcDNA3.1(+) expression

vector (Invitrogen) without the mesothelin insert (mock). The signal-to-background ratio of SS1P with A431.H9 cells was about 20-fold. Quantitation of mesothelin sites by flow cytometry with SS1P showed that each cell has about  $1 \times 10^5$  mesothelin molecules at the cell surface. The high expression level of mesothelin in A431.H9 cells was also confirmed by Western blot (data not shown). Cells were then incubated with sera from patients B115 and B41 and analyzed by fluorescence-activated cell sorting. Both sera reacted specifically with cells expressing mesothelin and not control cells. In addition, the signal was stronger with B115 than B41, as observed in ELISA (Fig. 4B).

To confirm that we were specifically detecting antibodies reacting with mesothelin, we also carried out an ELISA experiment in which we used a rabbit Fc-fusion protein made in 293T cells instead of mesothelin made in *E. coli*. On a plate coated with purified rabbit Fc-mesothelin fusion protein, the ELISA signals of patient sera B115, B41 and pooled normal sera at 1:100 dilutions were 0.26, 0.12, and 0.05, respectively (Fig. 4C). These values are in the similar range as the ELISA signals of B115, B41, and pooled normal sera using purified mesothelin proteins made in *E. coli*: at 1:100, dilutions the values were 0.6, 0.3, and 0.05, respectively (Fig. 2C). The slightly higher background and lower sensitivity in the ELISA experiments using rabbit Fc-mesothelin fusion proteins may be caused by its Fc portions or other experimental factors. Nevertheless, these data

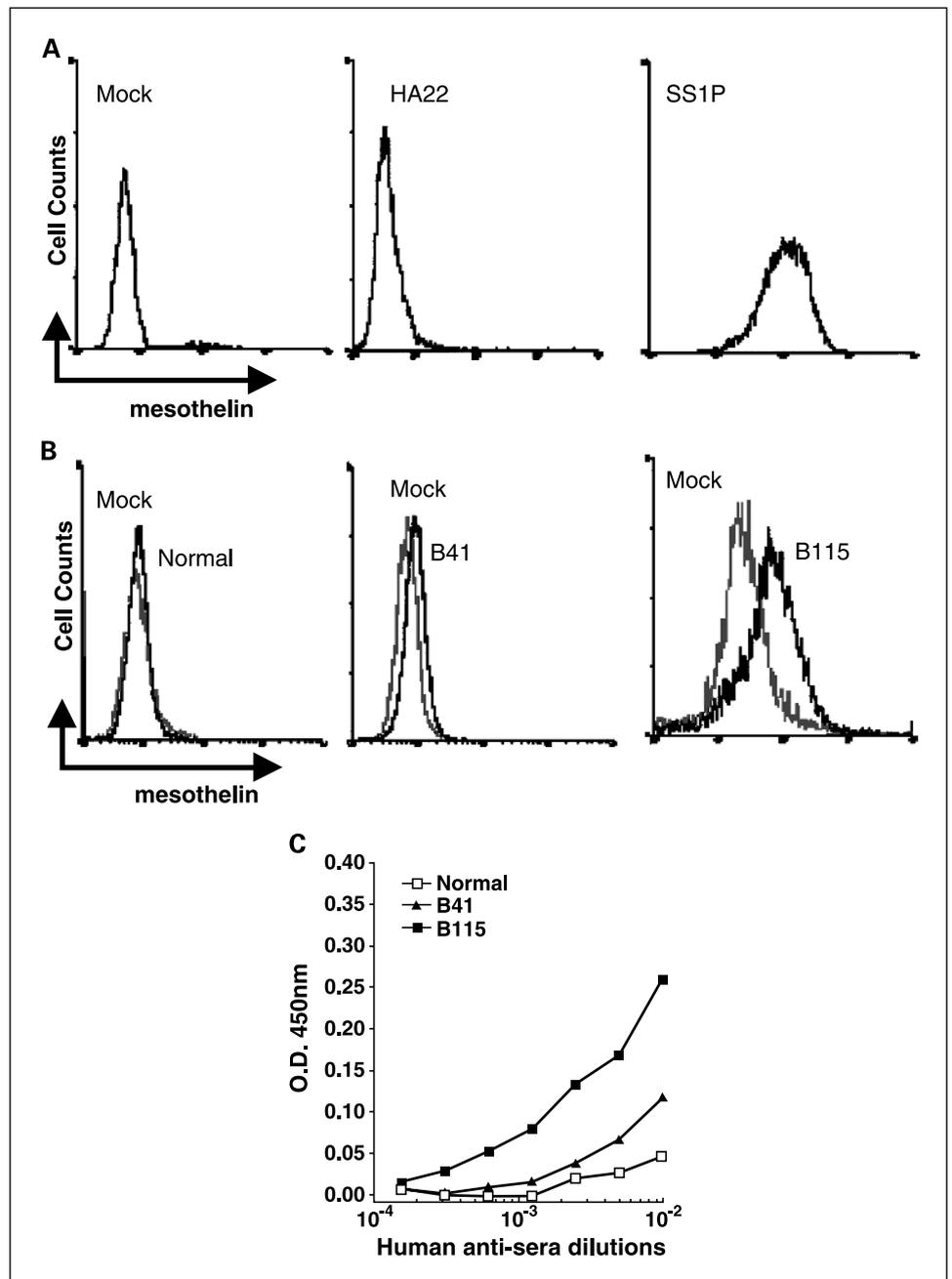
**Table 1.** Anti-mesothelin humoral immune response in relationship to mesothelin expression by tumors

Tumor type (samples examined)	Mesothelin expression		Anti-mesothelin antibodies
	Immunohistochemistry*	<i>n</i>	ELISA positive (%)
Mesothelioma (62)	+	49	26 (53)
	-	13	1 (8)
Ovarian cancer (24)	+	18	10 (56)
	-	6	0 (0)

\*Immunohistochemistry positive:  $>30\%$  of cells stained.

**Fig. 4.** Reactivity of patient sera against membrane-bound and soluble mesothelin proteins from mammalian sources.

**A**, immunotoxin SS1P bound to the A431 cell line (A431.H9) stably expressing mesothelin (*right*) but not the A431 cells transfected with the pcDNA3.1 vector with no insert (*mock*; *left*). Anti-CD22 immunotoxin HA22 did not bind A431.H9 cells (*middle*). **B**, flow cytometric analysis of two representative patient sera (B41, B115) bound to A431.H9 cells. Normal refers to the pooled sera from blood donors. **C**, ELISA analysis of pooled normal sera (*Normal*) and two representative patient (B41 and B115) sera bound to rabbit Fc-mesothelin fusion proteins.



confirm that the reactivity of patient sera against mesothelin from both bacterial and mammalian sources is significantly higher than normal sera.

## Discussion

In the present study, we developed a sensitive ELISA to detect mesothelin antibodies in human sera and showed that mesothelin-specific immunoglobulin G antibodies were detected in the sera of patients with mesothelioma and ovarian cancer but not in a normal healthy population. The results suggest that serologic recognition of mesothelin is cancer related and mesothelin is a new tumor antigen in patients with mesothelioma and ovarian cancer.

The generation of autologous antibodies against tumor differentiation antigens can be regarded as a signal that indicates the presence of the tumor in the host (reviewed in ref. 17). Interestingly, in this study, we found that significant serologic reactivity to mesothelin in patients was associated with high mesothelin expression in the tumor cells: 53% to 56% of patients with mesothelin immunostaining-positive mesothelioma and ovarian cancer were found to have antibody specific for mesothelin. NY-ESO-1 is one of the most immunogenic human tumor antigens defined to date. Antibodies to NY-ESO-1 were found in 40% to 50% of patients with advanced NY-ESO-1-expressing tumors (14). Our preliminary observations need to be validated in larger case/control studies. More patients must be analyzed to determine whether the levels

of antibodies to mesothelin are correlated with clinically relevant features such as volume of the tumor, type of tumor, or stage of disease.

The antibodies against mesothelin detected by our ELISA system are of the immunoglobulin G isotype, implying that cognate helper T-cell immunity might be present and operative in patients with a respective B-cell response. In pancreatic cancer patients who were vaccinated with irradiated tumor cells, Jaffee et al. observed that patients had a strong anti-mesothelin T-cell immune response (7). These results, together with our observation suggest that an integrated immune response against mesothelin may exist that involves both CD8<sup>+</sup> and CD4<sup>+</sup> T cells, as well as B cells.

Other tumor antigens such as NY-ESO-1 were first identified by using serologic analysis of recombinant cDNA expression library, an antibody-based approach and subsequently were shown to be targets of specific T-cell immunity in those individuals who had developed specific B-cell responses (18).

Similarly, antigens such as MAGE-1 that were first identified by cytotoxic T cells (19) also elicited humoral immunity (14) and can be identified by using serum antibodies from cancer patients (20). Our study may suggest that like the tumor antigens NY-ESO-1 and MAGE-1, mesothelin elicits both a B-cell as well as a T-cell immune response in some patients with tumors that highly express mesothelin. These results support ongoing clinical efforts towards developing immune-based therapies targeting mesothelin.

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